Selective Medium for Isolation of *Fusarium* Species and Dematiaceous Hyphomycetes from Cereals

STUART ANDREWS[1]* AND JOHN I. PITT[2]

*School of Chemical Technology, South Australian Institute of Technology, Adelaide, South Australia 5000,[1]
and Division of Food Research, Commonwealth Scientific and Industrial Research Organisation, North Ryde,
New South Wales 2113,[2] Australia*

A selective medium containing 2 µg of dichloran per ml, 200 µg of chloramphenicol per ml, and 1.5% bacteriological peptone was developed for the isolation of *Fusarium* species and dematiaceous hyphomycetes from cereals. The medium, designated DCPA, was shown to select against species of *Aspergillus*, *Penicillium*, *Cladosporium*, and mucoraceous fungi. DCPA was evaluated for use as an enumeration medium and compared satisfactorily with dichloran-rose bengal-chloramphenicol agar when both media were tested with a range of cereal samples. *Fusarium* species and dematiaceous hyphomycetes produced well-formed colonies with good conidial production on DCPA, permitting rapid identification of such isolates on this medium.

Many cases of mycotoxicoses have been attributed to the consumption by livestock of stockfeed prepared from grains infected with mycotoxigenic fungi (8, 9, 12, 14). The serious economic consequences associated with fungal growth in grains has provided an added incentive to determine the potentially mycotoxigenic flora of the raw materials to be incorporated into animal feeds.

To facilitate the screening of cereals and feedstuffs for potentially mycotoxigenic fungi, the use of selective media is desirable. For example, *Aspergillus flavus* and *Aspergillus parasiticus* can be detected and enumerated on *Aspergillus flavus* and *parasiticus* agar (13). *Penicillium viridicatum* on cereals can be detected on pentachloronitrobenzene-rose bengal-yeast extract-sucrose agar (6). Nash and Snyder (10) described a selective medium for *Fusarium* species with a peptone base and containing 0.1% pentachloronitrobenzene (PCNB) as a fungal inhibitor. This medium is highly inhibitory, producing *Fusarium* colonies which are scant and not distinctive. PCNB (0.2% final concentration) has also been used in a potato-based medium supplemented with inorganic salts to isolate fusaria from barley and malt (4). Although these media are highly selective for some *Fusarium* species, they are markedly inhibitory of others with PCNB present in excess as a saturated solution. PCNB is undesirable as a constituent of media because it has been reported as potentially carcinogenic (5).

It has been shown (7) that dichloran (2,6-dichloro-4-nitroaniline) is a potent fungal inhibitor and an effective substitute for PCNB at very low concentrations (2 mg/kg). Burgess and Liddell (2) described a modified Czapek yeast extract medium supplemented with dichloran (25 mg/kg) on which some fusaria produced distinctive colonies. In our experience the concentration of dichloran used in that medium is highly inhibitory to some *Fusarium* species and to many dematiaceous hyphomycetes. If the dichloran concentration is reduced, the high nutrient status of Czapek yeast extract agar promotes vigorous growth of *Aspergillus* and *Penicillium* species, and the selectivity of the medium is lost.

This paper describes the development and assessment of a dichloran modification of the Nash and Snyder medium which is selective for the growth of *Fusarium* species and for

many of the dematiaceous hyphomycetes as well. This medium, dichloran-chloramphenicol-peptone agar (DCPA), is not strongly inhibitory and permits the formation of distinctive colonies of these fungi, while at the same time selecting against *Aspergillus*, *Penicillium*, and similar genera.

MATERIALS AND METHODS

Media. The new medium described and assessed here, DCPA, has the following formulation: bacteriological peptone (Oxoid Ltd., London, England), 15 g; KH$_2$PO$_4$, 1 g; MgSO$_4$ · 7H$_2$O, 0.5 g; chloramphenicol, 0.2 g; dichloran (0.2% solution in ethanol), 1 ml (equivalent to 2 µg/ml); agar, 20 g; distilled water to 1 liter. The medium was sterilized at 121°C for 15 min; the final pH was 6.2.

In studies to develop the optimum formulation for DCPA, a basal medium, peptone agar, was prepared, consisting of bacteriological peptone, 15 g; K$_2$HPO$_4$, 1 g; MgSO$_4$ · 7H$_2$O, 0.5 g; agar, 20 g; distilled water to 1 liter. To this basal medium, dichloran (2 µg/ml) or chloramphenicol (200 µg/ml) was added to prepare dichloran-peptone agar and chloramphenicol-peptone agar, respectively. Modifications to the formulation of DCPA were prepared with concentrations varying from 0 to 25 µg of dichloran per ml, 0 to 1.0 g of MgSO$_4$ · 7H$_2$O per liter, and 5 to 30 g of bacteriological peptone per liter. All media were sterilized at 121°C for 15 min; final pH values were within the range of 6.0 to 6.4.

Media used for comparison with DCPA were dichloran-chloramphenicol-malt extract agar, potato-glucose agar (PDA), and dichloran-rose bengal-chloramphenicol agar (DRBC) (7). Dichloran-chloramphenicol-malt extract agar consisted of malt extract, 1%; agar, 2%; chloramphenicol, 200 µg/ml; and dichloran, 2 µg/ml (pH 5.3). PDA was made according to the formulation of Booth (1).

Inocula. The media were assessed by inoculation with cereal grains, using both dilution and direct plating. Samples of wheat, barley, oats, triticale, sorghum, lupins, peas, and soybeans were obtained from stockfeed manufacturers. For dilution plating, samples (10 g) were added to 0.1% peptone water (90 ml) and homogenized for 2 min in a Colworth stomacher. Samples were serially diluted 1:10 in 0.1% peptone water and then spread plated, 0.1 ml per plate in duplicate. For direct plating, samples (approximately 50 g)

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* Corresponding author.

TABLE 1. Comparison of growth rates of *F. graminearum* and *A. tenuis* on PDA, DCPA, and other peptone-based media after 5 days at 25°C

Fungus	Colony diam (mm) (avg and range) on:				
	Peptone-base agar	Dichloran[a]-peptone agar	Chloramphenicol[b]-peptone agar	DCPA[a]	PDA
A. tenuis[c]	31 (29–33)	30 (28–31)	29 (26–31)	30 (28–32)	57 (54–63)
F. graminearum[d]	42 (39–49)	38 (28–43)	38 (29–42)	33 (27–43)	59 (44–67)

[a] Dichloran (2 μg/ml).
[b] Chloramphenicol (200 μg/ml).
[c] Data from 10 isolates.
[d] Data from 11 isolates.

were surface disinfected with a 0.35% chlorine solution for 2 min, rinsed once with 100 ml of sterile water, and plated, 10 grains per plate, onto the specified media. A minimum of 50 grains per medium per sample was plated.

Pure culture inocula. The following isolates were used to assess growth and sporulation on some or all of the media: *Fusarium equiseti* FRR 2556 (FRR denotes the culture collection of the Division of Food Research, Commonwealth Scientific and Industrial Research Organisation, North Ryde, New South Wales, Australia), *F. acuminatum* FRR 2702, *F. oxysporum* FRR 2734, *F. moniliforme* FRR 2703, *F. solani* FRR 2197, *F. chlamydosporum* FRR 2847, *F. sporotrichioides* FRR 2552, *F. graminearum* FRR 2701, *F. semitectum* FRR 2738, *F. avenaceum* FRR 2848, *Cladosporium cladosporioides* FRR 3148, *P. viridicatum* FRR 3149, *Alternaria tenuis* FRR 3085, FRR 3087, FRR 3088, *A. tenuissima* FRR 3086, *Drechslera sorghicola* FRR 3094, *D. rostrata* FRR 3092, FRR 3093, *Curvularia lunata* FRR 3091, *C. eragrostidis* FRR 3089, *C. inequalis* FRR 3090, *Ulocladium chartarum* FRR 3095, FRR 3096, *Aspergillus flavus* FRR 3150, *Rhizopus stolonifer* FRR 3151, *Mucor hiemalis* FRR 3152.

Plates were inoculated at three points with spore suspensions dispersed in 0.05% Tween 80. To inoculate grain samples, spores were scraped from plates with an inoculating needle and added to the sample and 0.1% peptone water before stomaching. All plates were incubated in an alternating light and temperature regime consisting of 12 h in the presence of both daylight and UV lights at 25°C, followed by a 12-h dark period at 20°C (2). All plates were examined at 4 days and described or measured at 5 days.

RESULTS

Evaluation of DCPA for growth of *F. graminearum* and *A. tenuis*. Preliminary studies were carried out on a number of cultures of *F. graminearum* and *A. tenuis* isolated from weather-damaged wheat and sorghum samples. The growth of these two fungi on DCPA, dichloran-peptone agar, chloramphenicol-peptone agar, or peptone agar was compared with their growth on PDA. The inclusion of dichloran had no obvious effect on the rate of growth (Table 1) or the degree of conidiogenesis of *A. tenuis* on peptone-based media. However, mycelial growth of *A. tenuis* on DCPA was markedly restricted in comparison with that on PDA, and conidiogenesis was much heavier. Isolates of *F. graminearum* also produced smaller colonies with heavier conidiogenesis on DCPA (Table 1) compared with PDA. Both chloramphenicol and dichloran restricted radial growth of *F. graminearum*, while the inclusion of dichloran stimulated conidiogenesis. Both fungi produced much less pigmentation on DCPA than on PDA.

Effect of dichloran concentration on growth. Table 2 shows the effect of dichloran concentration on the growth of nine *Fusarium* species and 15 isolates of dematiaceous hyphomycetes. Inhibition of growth by dichloran was reported as the percentage of growth on peptone medium without added dichloran. At 2 μg/ml, dichloran restricted colony diameters of dematiaceous hyphomycetes by 15 to 27% while restricting the fusaria by only 7%. At 5 μg of dichloran per ml, colony diameters of dematiaceous hyphomycetes were inhibited by up to 50%, and those of fusaria were inhibited by 30%. At this and higher concentrations of dichloran, conidiogenesis was delayed or inhibited. On the basis of these observations, 2 μg of dichloran per ml was chosen as the optimal concentration, providing a good balance between reduction of mycelial growth, enhancement of conidiogenesis, and selection against other fungal species.

Effect of peptone and MgSO₄ concentrations on growth and conidiogenesis. Variations of DCPA were prepared with the following concentrations of MgSO₄ · 7H₂O: 0, 0.25, 0.50, 0.75, and 1.0 g/liter, and separately inoculated with 15 isolates of various species of *Fusarium*, *Alternaria*, *Curvularia*, and *Drechslera*. After incubation for 5 days at 25°C, radial growth, development of aerial mycelium, and conidiogenesis were measured. For all four genera studied, there were no obvious differences in any of the parameters examined as a result of variation in the concentration of $MgSO_4 \cdot 7H_2O$.

Using the same fungi, we studied the effect of variation of

TABLE 2. Inhibition of radial growth of Fusarium species, dematiaceous hyphomycetes, and other fungi by dichloran[a]

Fungus	Isolates tested	Colony diam (mm) in absence of dichloran	Inhibition (%) due to dichloran concn (μg/ml) of:			
			2	5	10	25
Fusarium spp.	9	47	7	30	66	94
Alternaria spp.	6	33	15	32	71	83
Drechslera spp.	3	47	21	50	87	96
Curvularia spp.	3	32	27	47	85	94
Ulocladium chartarum	2	35	24	46	80	83
Aspergillus flavus	1	27	11	19	30	44
Penicillium viridicatum	1	15	6	26	40	73
Cladosporium cladosporioides	1	14	18	46	57	61
Rhizopus stolonifer	1	>60	>85	100	100	100

[a] Colony diameters were measured after 5 days of incubation at 25°C; inhibition was measured as percent decrease in colony diameters compared with those in the absence of dichloran.

TABLE 3. Comparison of viable counts of fungi from cereals on DCPA and DRBC

Cereal	Viable count/g on:		Dominant mycoflora
	DCPA	DRBC	
Wheat	1.0×10^2	4.1×10^3	*Penicillium* spp.
Peas	3.5×10^3	1.8×10^4	*A. flavus*
Sorghum 1	6.5×10^2	8.8×10^3	*Penicillium* spp., *A. niger*
Sorghum 2	5.5×10^2	3.4×10^3	*C. cladosporioidies*
Triticale 1	1.2×10^5	2.3×10^7	*M. hiemalis, A. flavus*
Triticale 2	1.5×10^6	8.0×10^6	*A. flavus, M. hiemalis*
Triticale 3	8.5×10^4	1.5×10^7	*M. hiemalis*
Triticale 4	3.0×10^6	6.0×10^6	*A. flavus, A. terreus*
Oats	2.7×10^4	2.4×10^4	*Cladosporium* spp.
Triticale 5	1.1×10^3	6.0×10^2	*Cladosporium* spp.
Triticale 6	1.1×10^3	6.0×10^2	*Alternaria* spp.

peptone concentration (0.5, 1.0, 1.25, 1.5, 1.75, 2.0, and 3.0%) on growth and conidiogenesis. *Fusarium* and *Drechslera* isolates produced colonies on 0.5% peptone which were approximately 10% larger than those observed on 1.5% peptone, the concentration in DCPA. Colonies on 3.0% peptone were uniformly smaller than those produced on DCPA, and the general trend was that as the peptone concentration increased, the diameter of the colonies decreased, concomitant with an increase in aerial mycelium. Colonies produced by *Alternaria* and *Curvularia* species showed little difference as a result of variations in peptone concentrations.

In general, colonies produced on 0.5 and 1.0% peptone were thin and sparse with a substantial proportion of the mycelium subsurface. In addition, the lowest concentrations of peptone supported only moderate conidiogenesis. A concentration of 1.5% peptone was adopted, supplemented with 0.5 g of $MgSO_4$ per liter.

Comparison of fungal counts from cereals on DCPA and DRBC. To compare DCPA and DRBC as enumeration media for fungi on cereals, 28 samples were examined. Representative results from 11 samples are shown in Table 3. In nearly all samples the predominant mycoflora consisted of mucoraceous fungi, *Aspergillus*, *Penicillium*, and *Cladosporium* species. The counts produced on DRBC were usually slightly higher than those obtained on DCPA. To obtain further comparisons, some samples were artificially inoculated, and additional dilution plate counts performed (Table 4). In samples inoculated with *Fusarium* and *Alternaria* species only, the counts on DCPA were similar to or slightly higher than those on DRBC. In all samples inoculated with *Aspergillus*, *Penicillium*, and *Mucor* species, the counts on DRBC were marginally higher. The sample inoculated with *Cladosporium* species had a much higher count on DRBC than on DCPA.

DCPA as an isolation medium. In the screening of weather-damaged cereals for potentially mycotoxigenic fungi, samples were surface distinfected with chlorine (0.35%) and then direct plated onto DCPA. Each sample was scored for percentage of grains infected with particular fungi. The efficacy of the medium was determined by the number of genera producing distinctive colonies that enabled presumptive identification. A total of 140 samples were screened, comprising wheat (30), barley (10), oats (8), sorghum (33), triticale (46), lupins (5), peas (5), soybean (1), and sunflower seeds (2). The following genera were identified by direct examination of the DCPA plates: *Alternaria*, *Fusarium*, *Drechslera*, *Curvularia*, *Ulocladium*, *Aspergillus*, *Penicil-*

lium, *Scopulariopsis*, *Epicoccum*, *Phoma*, and *Chaetomium*.

DISCUSSION

Traditionally, species of *Fusarium* and dematiaceous hyphomycetes have been isolated on starch-based media, such as PDA, or malt agar, on which these fungi grow rapidly. Aerial mycelium is abundantly produced, but sporulation is moderate at best and often weak or absent. Moreover, mucoraceous fungi, aspergilli, and penicillia grow well on these media. Media more selective for *Fusarium* and dematiaceous hyphomycetes have been formulated with high concentrations of inhibitors or slowly assimilated carbon sources or both (2, 4, 10). However, in our experience these media are generally too selective, permitting the growth of only some species of *Fusarium* and few dematiaceous hyphomycetes. Fungi that do grow produce very dense compact colonies with poor sporulation, so that subculturing onto other media is necessary before isolates can be characterized.

DCPA was produced by modifying one of these formulations, the Nash and Snyder peptone medium (10), by replacing the high concentration of PCNB with a low level of dichloran and by using chloramphenicol in place of streptomycin plus neomycin. DCPA supports good growth of all the common fusaria and dematiaceous hyphomycetes, such as *Alternaria*, *Drechslera*, and *Curvularia* species, which produce well-formed colonies with good conidial production. An important consideration is that conidia are generally characteristic of those formed on standard identification media (3, 11), so that many isolates of *Fusarium* and other genera can be identified directly from the DCPA plates (S. Andrews and A. D. Hocking, manuscript in preparation). DCPA is also an effective isolation medium for *Fusarium* and dematiaceous hyphomyctes from commodities such as cereals, as these genera grow and sporulate well on DCPA, and it is selective against other common genera. Mucoraceous fungi are suppressed by dichloran, while the absence of a carbohydrate source is selective against *Aspergillus* and *Penicillium* species.

Although the inhibition of aspergilli, penicillia, and mucoraceous fungi by dichloran has been reported in detail (7), this is the first report on the effect of dichloran on *Fusarium* species and dematiaceous hyphomycetes. Burgess and Liddell (2) recommended 25 μg of dichloran per ml in their Czapek-based selective medium for *Fusarium* species. However, we found that only *F. graminearum* and occasionally *F. semitectum* and *F. sporotrichioides* will grow at this dichloran concentration on peptone-based media in 5 days. Dematiaceous hyphomycetes either do not grow or produce

TABLE 4. Comparison of viable counts obtained on DCPA and DRBC from inoculated cereal samples

Cereal	Dominant mycoflora	Viable count/g on:	
		DCPA	DRBC
Wheat 1	*F. chlamydosporum*	8.4×10^4	1.0×10^2
Wheat 2	*A. tenuis*	3.4×10^4	5.0×10^2
Wheat 3	*A. tenuissima*	4.3×10^4	5.5×10^3
Wheat 4	*Alternaria* spp.	1.8×10^5	1.2×10^5
Wheat 5	*Fusarium* spp.	2.6×10^7	1.9×10^7
Wheat 6	*Aspergillus* spp.	1.4×10^7	1.7×10^7
Wheat 7	*Penicillium* spp.	3.6×10^7	5.7×10^7
Wheat 8	*Cladosporium* spp.	1.0×10^2	6.6×10^5
Soybean 1	*M. hiemalis*	1.4×10^7	1.8×10^7

sterile microcolonies. *Fusarium* species and the dematiaceous hyphomycetes appear to be more sensitive to dichloran than *Aspergillus* and *Penicillium* species but are less sensitive than mucoraceous fungi. Our studies indicate that 2 μg of dichloran per ml is the optimal concentration for a peptone-based medium for the isolation of *Fusarium* species and dematiaceous hyphomycetes.

In comparative studies on the enumeration of naturally contaminated grain samples reported here, counts on DRBC were slightly greater than those on DCPA. However, neither *Fusarium* species nor dematiaceous hyphomycetes, for which DCPA was designed, were predominant in these samples. When inoculated grain samples were enumerated on both media, the counts on DCPA were higher than those obtained on DRBC for samples inoculated with fusaria and dematiaceous hyphomycetes. The reverse applied for samples inoculated with *Cladosporium*, *Mucor*, *Aspergillus*, and *Penicillium* species. In most cases DCPA alone would be satisfactory for the enumeration of fungi in cereals, although conidiogenesis may be slow in some potentially toxigenic aspergilli and penicillia and hence delay their recognition.

The significance of dilution plate counts obtained on DCPA or DRBC for homogenized grain samples is uncertain. This method enumerates the number of fungal propagules contaminating the outside of the grain as well as those infecting the grain. The recovery of *Alternaria* propagules by dilution plating is generally low, despite the fact that grain that is surface disinfected and direct plated onto DCPA will frequently show high-percentage infection rates with *Alternaria* species. For example, in Table 3, the samples sorghum 2, oats, and triticale 5 (Table 3) showed 94, 98, and 98% infection rates with *Alternaria* species, respectively, by direct plating on DCPA but did not yield any *Alternaria* colonies on dilution plates on either DCPA or DRBC.

In the examination of grain samples for potentially mycotoxigenic fungi, surface disinfection followed by direct plating onto DCPA is recommended, as it indicates the distribution of fungi infecting the grain. It should be noted that *Fusarium* species and dematiaceous hyphomycetes isolated on DCPA should not be maintained on this medium for more than a few days as the metabolism of peptone leads to the toxic accumulation of ammonia (11).

LITERATURE CITED

1. **Booth, C.** 1971. Fungal culture media. Methods Microbiol. **4:**49–94.
2. **Burgess, L. W., and C. M. Liddell.** 1983. Laboratory manual for *Fusarium* research. University of Sydney, Sydney, Australia.
3. **Ellis, M. B.** 1971. Dematiaceous hyphomycetes, p. 403–498. Commonwealth Mycological Institute, Kew, Surrey, United Kingdom.
4. **European Brewing Committee.** 1981. EBC analytica microbiologica: part II. J. Inst. Brew. **87:**303–321.
5. **Fairchild, E. J., R. S. Lewis, and R. L. Tatken (ed.).** 1977. Registry of toxic effects of chemical substances. U.S. Department of Health, Education and Welfare, Cincinnati, Ohio.
6. **Frisvad, J. C.** 1983. A selective and indicative medium for groups of *Penicillium viridicatum* producing different mycotoxins in cereals. J. Appl. Bacteriol. **54:**409–416.
7. **King, A. D., A. D. Hocking, and J. I. Pitt.** 1979. Dichloran rose-bengal medium for enumeration and isolation of molds from foods. Appl. Environ. Microbiol. **37:**959–964.
8. **Leistner, L.** 1984. Toxigenic penicillia occurring in feeds and foods: a review. Food Technol. Aust. **36:**404–406.
9. **Moreau, C.** 1979. Mould, toxins and food. John Wiley & Sons, Ltd., Chichester, United Kingdom.
10. **Nash, S. M., and W. C. Snyder.** 1962. Quantitative estimations by plate counts of propagules of the bean root rot *Fusarium* in field soils. Phytopathology **52:**567–572.
11. **Nelson, P. E., T. A. Toussoun, and W. F. O. Marasas.** 1983. *Fusarium* species—an illustrated manual for identification, p. 5–18. The Pennsylvania State University Press, University Park, Pa.
12. **Pier, A. C.** 1981. Mycotoxins and animal health. Adv. Vet. Sci. Comp. Med. **25:**185–243.
13. **Pitt, J. I., A. D. Hocking, and D. R. Glenn.** 1983. An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus*. J. Appl. Bacteriol. **54:**109–114.
14. **Rodricks, J. V.** 1976. Mycotoxins and other fungal related problems. Adv. Chem. Ser. **149:**23–50.